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Haem-polypeptide interactions during cytochrome *c* maturation

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Abstract

Cytochrome *c* maturation involves the translocation of a polypeptide, the apocytochrome, and its cofactor, haem, through a membrane, before the two molecules are ligated covalently. This review article focusses on the current knowledge on the journey of haem during this process, which is known best in the Gram-negative bacterium *Escherichia coli*. As haem always occurs bound to protein, its passage across the cytoplasmic membrane and incorporation into the apocytochrome appears to be mediated by a set of proteinaceous maturation factors, the Ccm (cytochrome *c* maturation) proteins. At least three of them, CcmC, CcmE and CcmF, are thought to interact directly with haem. CcmE binds haem covalently, thus representing an intermediate of the haem trafficking pathway. CcmC is required for binding of haem to CcmE, and CcmF for releasing it from CcmE and transferring it onto the apocytochrome. The mechanism by which haem crosses the cytoplasmic membrane is currently unknown.

Keywords: *ccm* gene; Cofactor; Cytochrome *c* biogenesis; Haem trafficking; Membrane protein complex

1. Introduction

Maturation of *c*-type cytochromes involves the posttranslational events that are required for a cytochrome polypeptide to be linked covalently to its haem cofactor(s). These include the targeting of the protein and of haem to the correct subcellular compartment, the processing of targeting sequences, and the formation of thioether bonds between the haem vinyl groups and cysteines of the protein, which are part of the typical haem-binding sequence C-X-X-C-H.

Cytochromes of the *c*-type are found in eukaryotic organelles and in extracytoplasmic compartments of bacteria and archae. They are localised on the *p*-side of the membrane, i.e., the intermembrane space of mitochondria, the chloroplast lumen and the periplasm of bacteria. The last step of haem biosynthesis occurs on the *n*-side of the membrane, i.e., the mitochondrial matrix, the chloroplast stroma or the bacterial cytoplasm. It is well established that covalent attachment of haem takes place on the *p*-side of the membrane [1–4]. Therefore, the apocytochrome and haem must be translocated through the membrane beforehand.

It has been recognised that despite the similar requirements for cytochrome *c* maturation, three different systems have evolved for this process, which have been called systems I, II and III [3]. The three systems are outlined in Table 1.

Systems I and II have a partial overlap in that they comprise at least one membrane protein with a characteristic tryptophan (W)-rich motif (CcmC/CcmF and CcsA/ResC, respectively) and one membrane

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protein with two S/TPC motifs (DsbD/DipZ and CcsC/CcdA, respectively). Both motifs have been shown to be functionally important. The cysteines in the latter set of proteins are believed to transfer electrons across the membrane, which are used to keep the haem binding cysteines reduced and thus competent for haem binding [5–7]. The W-rich motif is believed to participate in haem transfer, as discussed below. It is sometimes difficult to decide from genome sequences whether an organism uses system I- or system II-cytochrome *c* maturation. A good indication may be the flanking regions of the WXXXWXD core of the W-rich sequence motifs (Table 1). An even better indication is the presence of CcmB or CcmE homologues in organisms using system I and of CcsB/ResB homologues in organisms using system II, respectively. These proteins are unique and exhibit characteristic consensus sequences that should allow finding homologues by sequence comparisons. For example, a *ccmE* homologue in the plant genome of *Arabidopsis thaliana* encodes a mitochondrial protein, indicating that in plant mitochondria most likely system I is used for cytochrome *c* maturation. Conversely, in *Archaeoglobus fulgidus*, two proteins with W-rich motifs most similar to those of CcmC and CcmF have been identified; however, the organism neither possesses the other *ccm* genes, nor does it contain a gene encoding a CcsB/ResB homologue. Thus, it is not clear which system archae use for cytochrome *c* maturation.

System III is used by mitochondria of fungi, vertebrates and invertebrates. Two proteins, cytochrome *c* haem lyase (CCHL) and cytochrome c_1 haem lyase (CC_1HL) have been identified, which catalyse the covalent attachment of haem to cytochrome *c* and c_1, respectively, in the mitochondrial intermembrane space. Although haem lyases share only about 35% amino acid sequence identity, they contain one to three CPV motifs in their N-terminal domain [8]. These motifs are thought to be involved directly in haem binding. From sequence comparisons there is no homology between the system III haem lyases and any of the components of system I or II. The mitochondrial haem lyases do not contain hydrophobic sequences that are characteristic for integral membrane proteins. Nevertheless, they have been reported to be associated with the inner mitochondrial membrane [8–11]. It is not known whether other

Table 1
Three systems for cytochrome *c* maturation

System (representative[a])	Protein	Characteristic sequence motifs
System I (*E. coli*)	DsbA, DsbB	CXXC
	DsbD/DipZ	CXXC and S/TPC
	CcmA	Walker-A and -B motifs
	CcmB	FXXDXXDGSL
	CcmC	WGXXWXWDXRLTS
	CcmD	
	CcmE	LPDLFR and LAKHDE
	CcmF	WGGXWFWDPVEN
	CcmG	WCXXC
	CcmH	LRCXXC
	CcmI/CycH[b]	
System II (*Synechocystis*/*Bacillus*)	CcsA/ResC	WXXXWXWDPKET
	CcsB/ResB	Y/FXS/TXW/YF/Y-X_{13}-C
	CcsC/CcdA	G-X_5-S/TPC-X_5-P and G-X_7-S/TPC
System III (*Saccharomyces cerevisiae*)	cytochrome *c* haem lyase (CCHL)	CPV
	cytochrome c_1 haem lyase (CC_1HL)	CPV

[aa]Further representatives containing genes for proteins involved in cytochrome *c* maturation: system I, α- and γ-proteobacteria, deinococci, archae (?, see text), plant and protozoal mitochondria; system II, β-, δ-, ε-proteobacteria, Gram-positive bacteria, cyanobacteria, chloroplasts; system III, fungal, vertebrate and invertebrate mitochondria.

[bb]In *E. coli* CcmI is the C-terminal half of CcmH, whereas in other species it is a separate protein that has also been named CycH.

membrane-bound proteins are involved in system III cytochrome *c* maturation, because haem lyase activity has not been observed with purified components.

The only common features between the three systems for cytochrome *c* biogenesis may be the association of maturation factors with the membrane and the presence of at least one sequence motif containing a strictly conserved cysteine. This is quite surprising, because the maturation system needs to interact with haem and with apocytochrome *c* that in all systems contains the conserved C-X-X-C-H haem-binding site. To better understand why the systems are so diverse and yet are responsible for the same type of reactions, it is critical to understand the mechanisms of haem transfer and attachment to the apocytochromes for each system separately.

In this review article I summarise more recent findings concerning the transfer and attachment of haem to apocytochrome *c* for system I. In this system, haem trafficking has become a particularly interesting subject for experimental studies due to the discovery of the haem chaperone CcmE, which can be isolated as an intermediate of the haem transfer pathway [12,13].

2. *Escherichia coli* genes and proteins involved in cytochrome *c* maturation

The first genes involved in cytochrome *c* maturation of system I were discovered independently in the α-proteobacteria *Rhodobacter capsulatus* [14–16] and *Bradyrhizobium japonicum* [17–19] and were subsequently found in many other α- and γ-proteobacteria (for review see [1,3,4]). For conciseness, I use in the following the *ccm* gene nomenclature proposed by Page and Ferguson [4] for all system I components. The alternative gene names are listed in Table 2. System I genes are often organised in clusters [1]. For example, in *E. coli* the cytochrome *c* maturation genes *ccmABCDEFGH* are organised in an operon [20–22]. The first two genes, *ccmA* and *ccmB*, encode the ATPase and membrane permease subunits, respectively, of an ABC-type transporter. This transporter was believed to export haem to the periplasm for a long time, but there is now evidence accumulating that CcmAB is not a haem exporter. First, a haem reporter assay, in which periplasmic *b*-type cytochromes were analysed for haem binding, was used to test whether the *ccm* genes are required for a general haem transport to the periplasm. In *ccm*-defective strains haem-binding periplasmic *b*-type cytochromes could still be produced, whereas cytochrome *c* maturation was abolished [23,24]. Second, in *Paracoccus denitrificans* supplementation of growth media with haem did not stimulate *c*-type cytochrome formation in a *ccmA* or *ccmB* mutant [25]. Third, in vitro haem uptake into everted membrane vesicles was neither ATP dependent, nor was it different in a *ccmA* mutant compared with the wild type [26]. All these results would be unexpected if CcmAB were a haem exporter. Nevertheless, a role in haem transport for CcmAB cannot be ruled out completely on the basis of these findings.

Haem transfer to apocytochrome *c* requires that the cysteines of the haem-binding site in the protein be reduced. The redox reactions involved in cytochrome *c* maturation have been reviewed recently [27]. The last two genes of the *ccm* operon, *ccmG* and *ccmH*, are believed to transfer electrons from

Table 2
System I genes

Gene	Alternative gene names	Proposed function of gene product
ccmA	*helA*, *cycV*	ATP-binding subunit of ABC transporter
ccmB	*helB*, *cycW*	Permease subunit of ABC transporter
ccmC	*helC*, *cycZ*, *cytA*	Haem insertion into CcmE, haem export?
ccmD	*helD*, *cycX*, *cytB*	Assisting haem insertion into CcmE
ccmE	*cycJ*, *cytC*	Haem chaperone
ccmF	*ccl1*, *cycK*, *cytD*	Putative haem lyase
ccmG	*helX*, *cycY*, *tlpB*, *cytE*, *dsbE*	Reduction of CcmH
ccmH	*ccl2*, *cycL*, *cytF*	Reduction of cytochrome *c* haem binding site
ccmI	*cycH*, *cytG*	Recognition of apocytochrome *c*

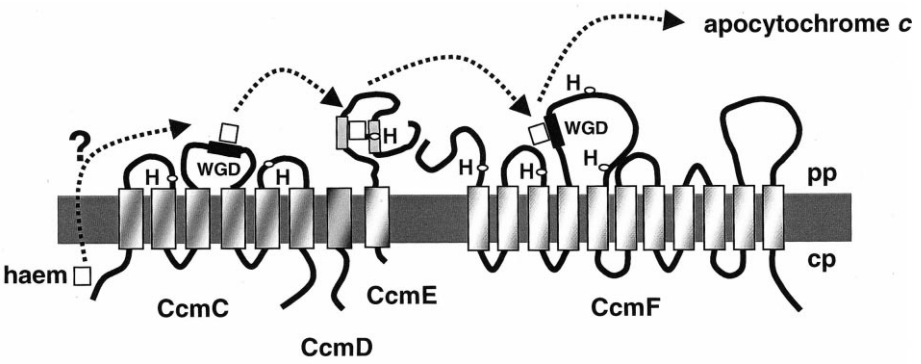

Fig. 1. Topology and conserved motifs of Ccm proteins involved in haem delivery to apocytochrome *c*. The postulated haem transfer is indicated by dotted arrows. H, invariant histidine; WGD, W-rich motif; pp, periplasm; cp, cytoplasm.

DsbD to the haem-binding site of *c*-type cytochromes, thus keeping the cysteines reduced. The free thiols of apocytochrome *c* can then form covalent thioether bonds across the vinyl double bonds of haem. DsbD is an integral membrane protein with the capacity to form intramolecular disulphide bonds. It transfers electrons from the cytoplasmic thioredoxin to the periplasm [28]. Various periplasmic thioredoxin-like proteins are thought to be reduced by DsbD, among them CcmG [7]. CcmG is believed to reduced CcmH, which then interacts with the haem-binding site of apocytochrome *c* [29–31]. This interaction prepares the apocytochrome *c* for haem binding.

3. The haem chaperone CcmE

A key role in system I cytochrome *c* maturation has been attributed to CcmE, the periplasmic haem chaperone that was isolated as an intermediate of the haem delivery pathway [12,13]. The haem-binding form of this protein had escaped detection for a long time, because it does not accumulate in the wild type. Only when CcmE is overproduced, or when later steps of cytochrome *c* maturation are blocked does it reach detectable levels. It was found that CcmE binds haem covalently. In vivo haem transfer experiments showed that release of haem from CcmE occurs only in the presence of apocytochrome *c* and also depends on CcmF, CcmG and CcmH [12,13,29]. Conversely, these proteins are not necessary for binding of haem to CcmE.

CcmE of *E. coli* is a 159 amino acid polypeptide with an N-terminal hydrophobic membrane anchor and a soluble, periplasmic domain (Fig. 1). This domain contains two well-conserved sequence motifs (Table 1), of which the second encompasses the haem-binding residue, a histidine (H130). Interestingly, haem binding also occurred when the soluble domain of CcmE was expressed, but only when CcmE was translocated to the periplasm [32]. This implies that both apo-CcmE and haem are exported to the periplasm before haem binding takes place.

The experimental evidence that haem is bound covalently to H130 is compelling; besides the classical haem stains for denatured proteins carrying covalently bound haem [13,32], perhaps the best indication was provided by mass spectrometry analyses of purified CcmE, which resulted in molecular masses corresponding to apo-CcmE and haem on the one side, and holo-CcmE on the other side [32]. Moreover, CcmE was digested with trypsin, and a haem-binding dodecamer peptide was isolated and subjected to N-terminal sequence analysis and tandem mass spectrometry. From the results of these experiments it was deduced that H130 was the haem-binding amino acid. This finding was extraordinary, because so far no other type of covalent haem binding in proteins than the thioether bonds in *c*-type cytochromes has been described. When H130 was changed to alanine, haem binding was blocked, and CcmE was produced exclusively in the apo form. In addition, cytochrome *c* maturation was impaired, indicating that haem-binding CcmE is an obligatory intermediate in the cytochrome *c* biogenesis pathway.

It was concluded that CcmE functions as a periplasmic haem chaperone by binding haem, shielding it from non-specific interactions with other proteins and lipids, and transferring it to the apocytochrome [32].

At a first glance it is not easy to understand why haem should be bound *covalently* by a polypeptide whose function is to release the cofactor in a next step and transfer it to a new target protein. It is possible that in this case not the strength of the haem histidine bond is critical, but rather the stereochemistry of the haem ligation to apocytochrome *c* (see below).

CcmE has also been investigated more closely in *B. japonicum* [19] and *R. capsulatus* [33]. In both organisms, *ccmE* mutants lead to lack of cytochrome *c* formation. Nevertheless, holo-CcmE was not found in either of them, probably because the transfer of haem from CcmE to its final receptor, the apocytochrome *c*, occurs rapidly and holo-CcmE never accumulates to detectable levels. In the *R. capsulatus* mutant an increase of porphyrin excretion was observed, indicating that haem delivery was impaired. When the *B. japonicum* CcmE was overproduced in *E. coli* it was capable to bind haem and it was functional in cytochrome *c* maturation, although at reduced levels. Upon mutation of the invariant histidine to alanine (H122A), haem binding and cytochrome *c* maturation were impaired (H. Schulz and L. Thöny-Meyer, manuscript in preparation). The invariant histidines of the *R. capsulatus* CcmE were also shown to be important for cytochrome *c* biogenesis [33]. In summary, these data support the idea that CcmE generally functions as a haem chaperone in type I cytochrome *c* maturation by binding haem in the periplasm, shielding it from non-specific interactions with proteins or lipids and transferring it to its receptor, the apocytochrome *c*.

4. CcmC is required for binding of haem to CcmE

CcmC is a membrane protein with six transmembrane helices that have been mapped topologically in the *R. capsulatus* and *Pseudomonas fluorescens* homologues [34,35] (Fig. 2). CcmC mutants have been described which are not only affected in cytochrome *c* maturation but also exhibit other phenotypes. Partic-

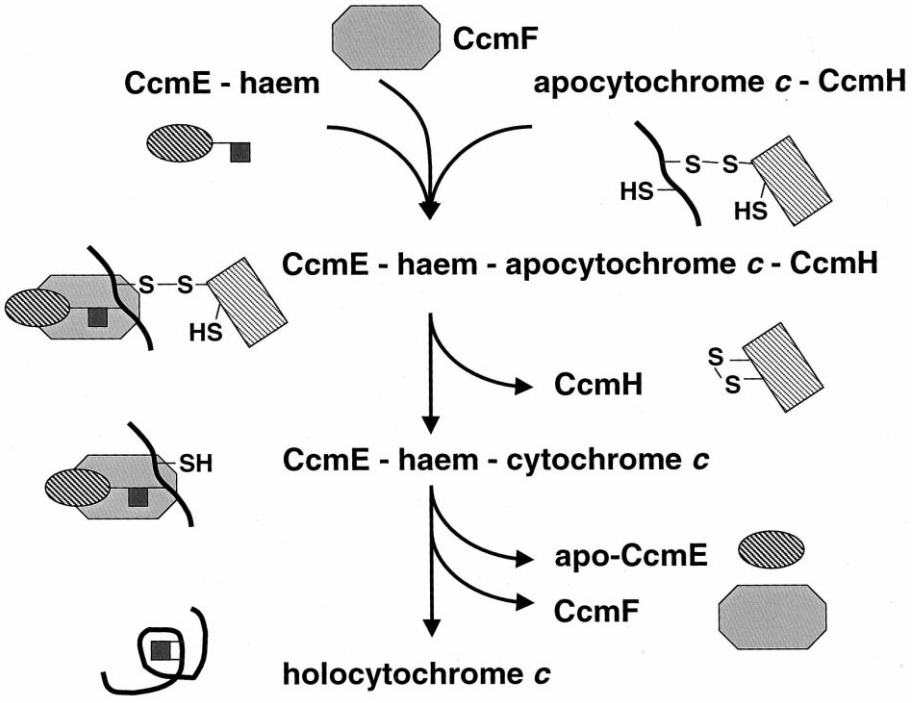

Fig. 2. A putative mechanism for haem delivery from CcmE to apocytochrome *c*. A sequential pathway for the formation of the two thioether bonds is proposed. The reductive recycling of CcmH by CcmG [29] is not shown.

ularly interesting was the analysis of mutants affecting the *P. fluorescens* CcmC, which is required for production and utilisation of pyoverdine, a high affinity fluorescent siderophore [35,36]. In *P. denitrificans*, a *ccmC* mutant in addition to defects in cytochrome *c* maturation and siderophore production showed intolerance to rich medium [37]. The connection between these processes is not known.

The best conserved sequence motif W-G-T/S-Φ-W-X-W-D-A/P-R-L-T (with Φ for W/F/Y) resides in the periplasmic loop domain between the third and fourth transmembrane helix of CcmC (Fig. 1) and has a high similarity to the W-rich sequences of CcmF and NrfE (see below) and the CcsA homologues of type II cytochrome *c* maturation (Table 1). The accumulation of hydrophobic amino acids within this motif has led to the speculation that it forms a platform for the binding of haem [1,3,15,38]. It was also noted that a W-F-W-D motif is found in hemopexin, which binds haem with a high affinity [39]. Moreover, the flanking periplasmic loops between transmembrane helices 1–2 and 5–6 (Fig. 1) carry an invariant histidine, which acts as an axial haem ligand in many cytochromes. The idea is that together with these two histidines the W-rich motif binds haem transiently. This idea was tested by site directed mutagenesis. Mutations in conserved residues of the W-rich motif led to partial or full loss of function of CcmC [34,35,43]. However, some point mutations in the *P. fluorescens* W-rich motif of CcmC affected pyoverdine production but not cytochrome *c* maturation, whereas others had an opposite effect, suggesting a dual function of CcmC in this organism. The importance of the conserved histidines for activity of CcmC was shown in *R. capsulatus* [34] and *E. coli* [12], whereas it is less clear in *P. fluorescens* [35].

The role of CcmC during cytochrome *c* maturation became more obvious when a *ccmC* deletion mutant was shown to be affected in the production of cytochrome *c* and also in haem attachment to CcmE [12]. In fact, CcmC appears to be the only factor that is required strictly in *E. coli* for the synthesis of haem-binding CcmE. This finding suggests that CcmC is either a haem transporter or catalyses the covalent attachment of haem to CcmE, or even exerts both functions.

The question of haem transport has been addressed using *ccmC* mutants. The haem reporter system using periplasmically expressed *b*-type cytochrome was used to test an in-frame Δ*ccmC* mutant. Haem was incorporated into periplasmic *b*-type, but not *c*-type cytochrome [24]. Therefore, if CcmC is involved in export of haem to the periplasm, yet another mechanism of haem transport across the membrane to reach periplasmic *b*-type cytochromes must be postulated.

The role of CcmC in the covalent attachment of haem to CcmE is likely because CcmE does not bind haem covalently when apo-CcmE and haem are mixed in the test tube (H. Schulz, unpublished results). If haem is bound to CcmE stereospecifically at one of the two vinyl groups, it is reasonable to assume the involvement of an enzyme, which helps to correctly position haem and apo-CcmE face to face and perhaps catalyses the formation of the haem-histidine bond. CcmC could then be described as a CcmE haem lyase.

CcmD, another membrane-bound subunit of the Ccm maturation apparatus, was shown to be involved in, although not essential for, ligation of haem to CcmE. This protein not only stabilises the CcmE polypeptide in the membrane [12], but apparently it also interacts with CcmC. This is postulated because the presence or absence of CcmD clearly affects the phenotypes of point mutations in the W-rich motif of CcmC [43]. CcmD is a small membrane protein with a hydrophobic N-terminal and a hydrophilic C-terminal half, whose topology was mapped with the C terminus residing in the cytoplasm [34] (Fig. 1). Since the W-rich motif of CcmC and the hydrophilic domain of CcmD are localised on opposite sides of the membrane, the proteins may interact directly via their transmembrane domains. The currently most attractive model to explain how haem is transferred to CcmE is depicted in Fig. 1 and involves a translocation via CcmC, whereby haem is bound transiently and non-covalently in a hydrophobic pocket that is formed by the W-rich motif, and liganded axially by two histidines. From there haem is donated to a binding site in CcmE: it approaches the invariant histidine of CcmE with one specified vinyl group, which is then bound covalently to the histidine.

5. Transfer of haem to apocytochrome *c*

The transfer and covalent attachment of haem to apocytochrome *c* is a key reaction of cytochrome *c* biogenesis. In system I, the haem donor is holo-CcmE. Haem transfer was shown to depend on CcmFGH [13,29,32], and more recently also on the ABC transporter CcmAB [12]. CcmG and CcmH are involved in activating the haem-binding site of the apocytochrome for ligation by keeping the cysteines reduced (for review see [27]). The function of the ABC transporter is unknown, as holo-CcmE can be formed in Δ*ccmA* and Δ*ccmB* mutants that overproduce CcmC, while cytochrome *c* maturation is blocked [12].

CcmF is an integral membrane protein with 11 transmembrane helices [34]. It may be the virtual haem lyase that is supposed to have an activity of ligating apocytochrome and cofactor by formation of two thioether bonds. Several lines of evidence support this hypothesis. (i) A CcmF mutant accumulates haem-binding CcmE, but is defective in the release of haem from CcmE. This suggests that the activity of CcmF is required after the one of CcmE in the cytochrome *c* maturation pathway. (ii) Like CcmC, CcmF and its homologues possess the W-rich motif, although with a slight variation (Table 1). In addition, four invariant histidines are found in domains of the protein that have been mapped to the periplasm where the W-rich motif resides [34]. Thus, like for CcmC, it can be speculated that CcmF interacts with haem. This would be in agreement with a function as a haem lyase. The postulated role of CcmF in periplasmic haem trafficking is depicted in Fig. 1. (iii) *E. coli* possesses a CcmF paralogue, NrfE, which is required for maturation of the pentahaem cytochrome c_{552} NrfA. NrfA is a periplasmic nitrite reductase with four classical haem-binding sites of the C-X-X-C-H type, to which haem is bound with the help of the *ccm* gene products. However, in the fifth haem-binding site, C-W-S-C-K, a lysine replaces the histidine that normally is an axial ligand of the haem iron. Intriguingly, haem is bound covalently to this alternative motif using the CcmF and CcmH paralogues NrfE and NrfG, respectively [40]. NfrE carries the same W-rich motif like CcmF [41]. Thus, it is reasonable to assume that CcmF and NrfE constitute part of the classical and of an alternative haem lyase, respectively, which releases haem from CcmE, transfers it to the apocytochrome and facilitates thioether bond formation. NrfE recognises the special NrfA haem-binding site that cannot be selected as a target for haem binding by CcmF. It is likely that CcmF co-operates with CcmH and NrfE with NrfG for the selection of the appropriate haem-binding site in the apocytochrome.

6. Summary

The mechanism(s) by which haem is transferred through biological membranes of mitochondria, chloroplasts and bacteria is not well understood. Yet, it is known that bacteria using extracellular haem as a source of iron or porphyrin possess specialised transport systems for haem acquisition, which include outer as well as inner membrane haem transporters [42]. This indicates that haem cannot diffuse readily through lipid bilayers. In an attempt to investigate haem export to the periplasm, haem uptake into everted membrane vesicles of *E. coli* has been measured recently [26]. It was found that haem associates rapidly with the membranes of the vesicles, and then is taken up slowly. However, it was not shown whether haem uptake into the vesicles includes the release of haem from the inner leaflet of the membrane vesicle, and whether it is dependent on a proteinaceous transporter. Clearly, it was not dependent on the presence of the ABC transporter CcmAB.

Deletion mutation of *ccmC* blocked haem binding to CcmE and subsequent cytochrome *c* maturation [12], but not the incorporation of haem into periplasmic cytochrome *b* [24]. At present, it cannot be ruled out that CcmC is a haem transporter specifically used for cytochrome *c* biogenesis. It is also possible that haem reaches the outer leaflet of the cytoplasmic membrane by diffusion and is then picked up by the putative haem-binding domain of CcmC, from where it can be delivered to CcmE.

The haem transfer from CcmE to apocytochrome *c* involves CcmF on the one hand and the CcmG/H redox thioredoxin-like system on the other hand. If haem is donated to cytochrome *c* in a stereospecific way, a scenario as presented in Fig. 2 might be considered. CcmE binds haem at one specific vinyl

group, leaving the other vinyl group accessible for an attack by a cysteine. In apocytochrome *c*, which is reduced by CcmH, one of the two cysteines forms at some point of the reduction reaction a mixed disulphide with CcmH. In this situation the other cysteine is free and can react with the haem vinyl group. Reduction of the mixed disulphide between apocytochrome *c* and CcmH leads to a CcmE-haem-cytochrome *c* intermediate, in which the free SH group of the haem-binding site displaces the haem-binding histidine of CcmE by forming the second thioether bond. The result of this reaction is free apo-CcmE and holo-cytochrome *c*. CcmF serves as a platform to bring CcmE and apocytochrome *c* in close contact, thereby promoting the ligation reactions. Even though the proposed mechanism is highly speculative, it can be tested experimentally. This requires the chemical characterisation of the haem-histidine bond, the trapping and isolation of short-lived intermediates, and studies on protein-protein interactions between CcmE, CcmF and CcmH.

Apparently, the presentation of haem for ligation by a specific haem chaperone is unique for system I cytochrome *c* maturation and may have evolved only in certain bacteria, perhaps making cytochrome *c* formation more efficient. The minimal requirement for covalent haem ligation seems to be a reducing condition to guarantee the availability of thiols and the transport of haem through the membrane. These two requirements may be met by the two conserved characteristics between system I and II, i.e., a conserved cysteine and the W-rich motif. The precise role of the mitochondrial type III haem lyase is not clear at all, and further biochemical characterisation of this enzyme would be extremely helpful to better understand the critical steps of cytochrome *c* maturation.

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